

ELECTRON PROBE ANALYSIS OF CALCIUM TRANSPORT BY SMALL INTESTINE

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ABSTRACT

Calcium transport in small intestine of rat and chick has been studied at the cellular level using the electron probe X-ray microanalyzer. Tissues were examined directly after removal as well as after incubation in a calcium solution. In both preparations, discrete calcium localizations were found associated with intracellular and extracellular goblet cell mucus. The *in vitro* preparations showed calcium in transit across the absorptive epithelium in discrete localizations. Although the primary path of transport was along lateral cell borders, some localizations were found in the cytoplasm in a supranuclear position. The effect of vitamin D depletion and repletion was to decrease and increase, respectively, the number of calcium localizations in transit across the epithelium. These results suggest that calcium is transported while in a sequestered form and indicate that goblet cell mucus plays a role in this transport process.

The majority of studies on the absorption of calcium from intestinal lumen have employed whole tissue preparations or tissue homogenates; these studies yield answers averaged over a population of cells and cell types and therefore cannot answer several fundamental questions at the cellular level. Among these unanswered questions are the following: (a) are only absorptive cells of intestinal epithelium involved in the transport process? (b) are all absorptive cells equally and simultaneously involved in the transport process? (c) what is the path of calcium through transporting cells, e.g., is calcium constrained to specified cellular routes? and (d) what is the form of calcium movement across the epithelium, e.g., does it move down a continuous thermodynamic gradient, does the cell content increase homogeneously, or is calcium in some way sequestered within cellular compartments?

To answer these questions it is necessary to resolve the activities of individual intestinal cells of the mucosal epithelium. Electron probe microanalysis (EPMA) can meet this requirement and we have used it to examine the distribution of calcium within epithelial cells of the small intestine of rat and chick.

MATERIALS AND METHODS

Experiments were conducted with rats and chickens. Male Rochester Wistar rats were raised on a commercial diet (Purina Lab Chow) and unless otherwise specified were used when animal weight was 100 g. Male White Leghorn chicks were obtained from Babcock Poultry Farms (Ithaca, N.Y.) and raised on a commercial diet (Purina Chick Startena). Chicks from 4 days to 2 wk of age were used. Animals were allowed food and water ad libitum unless otherwise specified.

All animals were killed by decapitation; the abdominal cavity was immediately opened and the intestinal region

of interest quickly removed. Originally, the luminal contents were flushed with isotonic saline, however, similar to the experience of Florey (17), this procedure appeared to cause an increase in goblet cell mucus discharge and was discontinued.

To determine the *in vivo* distribution of calcium, tissue was examined from duodenum, jejunum, and ileum of young rat, from the duodenum of mature rat, from the duodenum of chicken, and from the duodenum of young rats deprived of food and water 24 h before sacrifice. Tubular segments 2-cm long were cut from the intestine, slit longitudinally, and placed immediately in a pool of buffered acrolein fixative containing 1% oxalate. The tissue was cut into small squares approximately 1 mm on a side and fixed, dehydrated, and embedded following the procedure of Coleman and Terepka (10, 11), a procedure utilizing oxalate to prevent calcium loss and redistribution.

To determine the calcium distribution under *in vitro* transport conditions, three adjacent 2-cm tubular segments were cut from the duodenum starting 0.5-cm distal to the pyloric valve. A control segment was fixed and embedded as outlined above. The remaining two segments were incubated following one of two procedures. Initially, the "uptake" procedure of Martin and DeLuca (25) was utilized. The incubation medium contained 125 mM sodium chloride, 10 mM fructose, 30 mM Tris buffer (Tris(hydroxymethyl)-aminomethane), pH 7.4, and 2 mM calcium chloride. Since active calcium transport saturates between 2 and 5 mM calcium (43), and at 2 mM with our particular experimental procedure (25), this molarity was chosen to maximize active transport while minimizing the passive component. The incubation medium was vigorously bubbled with oxygen, and temperature was maintained in a water bath. Incubation was for 5 or 15 min, after which the disk of tissue which had been exposed to the incubation medium was cut out, fixed, and embedded as before.

The procedure used later in the experiments is a modification of the above, which in our hands occasionally resulted in extensive damage to the exposed tissue. It was found that placing the unmounted tissue directly into the incubation medium minimized tissue damage and resulted in a calcium distribution identical to that observed with the previous technique. Similarly, the addition of 1 mM KCl to the incubation medium, added to assure the functioning of the cellular sodium-potassium pump, had no discernible effect on the calcium distribution.

For the vitamin D experiments, chicks were raised from hatching on a commercial rachitogenic diet (Nutritional Biochemicals Corp., Cleveland, Ohio). Chicks were utilized in their 6th or 7th wk, when they weighed 200 g and had serum calcium levels between 6 and 8 mg/100 ml. Vitamin D-treated chicks were prepared by oral administration of 500 IU of vitamin D₃ (General Biochemicals, Chagrin Falls, Ohio) dissolved in Wesson Oil to rachitic chicks 72 h before sacrifice.

Vitamin D-deficient male Holtzman rats were obtained from the laboratory of Dr. Hector DeLuca at the University of Wisconsin, Madison, Wisconsin. These rats were raised on diet no. 11 (15) and were utilized in their 7th wk, when they weighed 160 g and had serum calcium levels of 5.3 mg/100 ml. Vitamin D-treated rats were prepared by stomach intubation of 500 IU of vitamin D₃ in Wesson oil to vitamin D-deficient rats 40 h before decapitation. At the time of sacrifice the serum calcium levels of D-treated rats had recovered to 9.0 mg/100 ml. Animals were allowed food and water *ad libitum*.

To demonstrate calcium uptake with our *in vitro* transport conditions, tissue from normal rat duodenum was incubated as described except for the addition of ⁴⁵Ca to the incubation medium. At the end of incubation the tissue was rinsed in isotonic saline (room temperature) and briefly blotted with facial tissue to remove any surface droplets. The tissue was weighed and ashed overnight at 600°C. The residue was taken up in 0.1 N HCl and an aliquot counted in a liquid scintillation spectrometer.

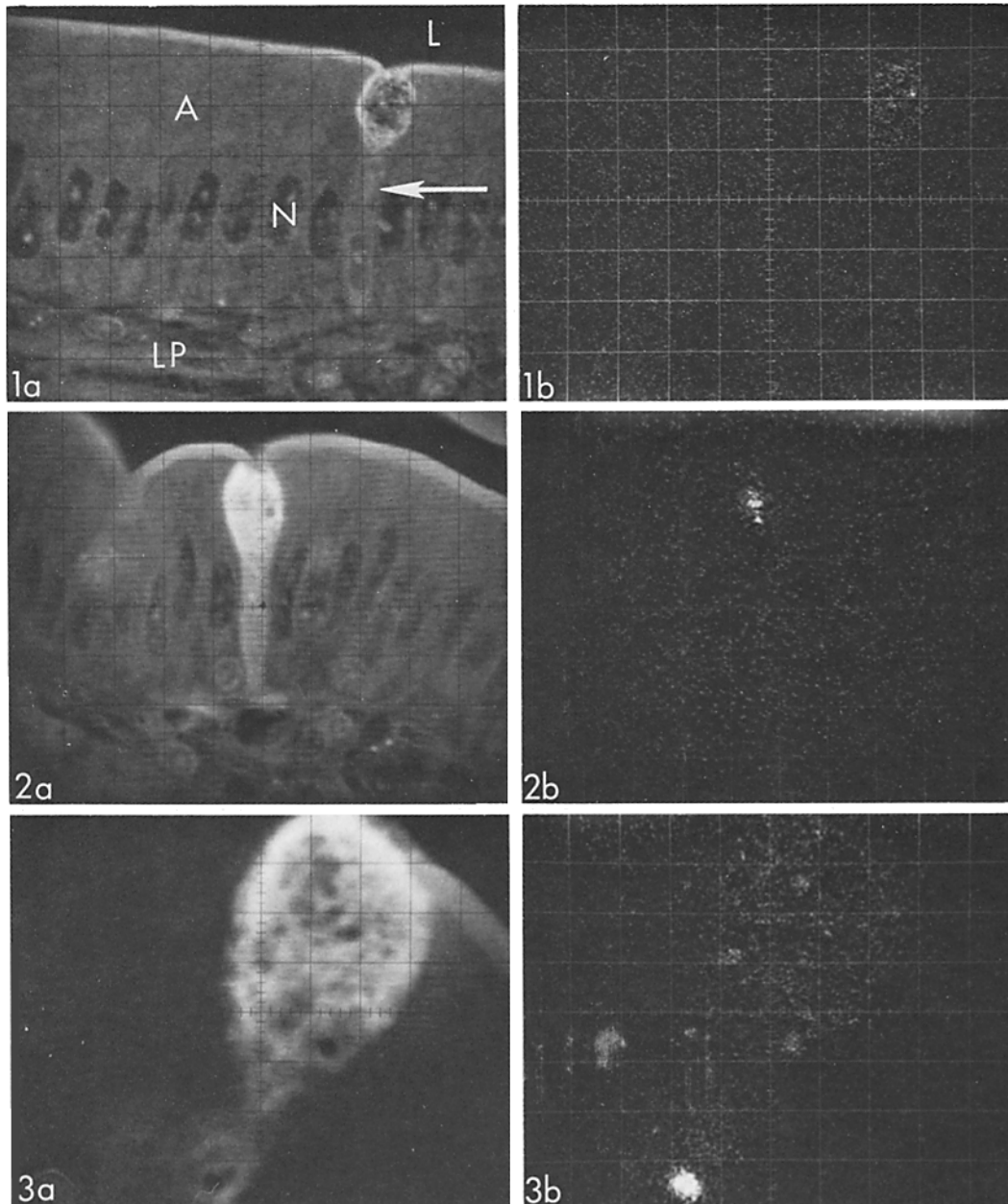
Thin sections of plastic-embedded tissue were prepared for electron microscopy and 1- or 2- μ m thick samples were cut dry with glass knives for electron probe analysis and were analyzed without an evaporated metal coating; specimen mounting techniques, analysis conditions, and data collection procedures have previously been described (10).

To measure concentrations of cytoplasmic calcium, the condenser lens was defocused, which enlarged the electron beam to a diameter approximately 0.5 the width of the apical absorptive cell. X-ray intensities from sections 1- μ m thick were integrated with a static electron beam for 5 min and corrected according to the BASIC procedures (Biological Analysis by Substrate Intensity Correction) (40, 41), with background measured off-peak (2).

RESULTS

Since EPMA is a relatively new technique, some discussion of data interpretation is pertinent. Fig. 1 *a* shows a microprobe sample current image of a portion of a villus from rat duodenum. Contrast in this image is a function of local atomic number differences. In this case, since the tissue was postfixed with osmium tetroxide during sample preparation, it is likely that contrast is largely due to the presence of this stain.

Fig. 1 *b* is the calcium K _{α} X-ray image showing the calcium distribution in the sample current picture of Fig. 1 *a*. The grids in these two figures cover identical areas, and the calcium distribution may be correlated with the sample current image by referring to the grid. Scanning images like these are impractical for detecting very low elemental



FIGURES 1-26 Figs. marked (a) are sample current images; corresponding figures marked (b) are calcium K_{α} X-ray images. The magnification of the microprobe pictures will be quoted with reference to the grid squares superimposed on the images.

FIGURE 1 A portion of a villus from rat duodenum. The lumen (L) of the intestine is at the top of the sample current image. The absorptive epithelial cells (A) contain prominent nuclei (N) containing white spots of condensed chromatin. The white band at the luminal surface is the brush border. An arrow points to a goblet cell whose apical end is swollen with accumulated mucin. This epithelium rests on the core of the villus, the lamina propria (LP). When this image is compared to the X-ray image, the calcium which exceeds background is seen to be associated with the region of goblet cell mucin accumulation. The scale for this image is $8 \mu\text{m}$ per side of the grid square (SGS). The picture height is $64 \mu\text{m}$.

FIGURE 2 A portion of a villus from rat ileum. The calcium exceeding background is confined to a small region within the goblet cell mucin. The scale is $7 \mu\text{m}/\text{SGS}$.

FIGURE 3 A portion of a goblet cell from rat ileum. Diffuse and discrete calcium distributions are observed associated with regions of mucin production and accumulation within the goblet cell. The scale is $2 \mu\text{m}/\text{SGS}$.

concentrations because background radiation or X-ray noise becomes prominent in X-ray images with long exposure times. For example, in Fig. 1 *b* which required 30 min of exposure, a random pattern of points appears over most of the X-ray image. This random distribution need not represent the presence of calcium since one can generate a similar random distribution if the X-ray spectrometer is not tuned to the calcium X-ray peak. Due to the inability visually to distinguish low concentrations from random noise, very low concentrations are best detected by integrating the X-ray intensity (counting rate) for long periods with a static beam as described under Materials and Methods.

I. Calcium Distribution In Vivo

The normal *in vivo* calcium distribution was investigated throughout the length of small intestine in rats and in the duodenum of chicks. The chick was not so extensively studied as the rat for several technical reasons. Epithelial cells in the chick are longer, making it more difficult to keep individual cells from apex to base within the plane of the section. Contrast is also less in the chick, and the lateral cell borders are less distinct. These characteristics made it more difficult to define the specific cellular calcium distribution in the chick, but it was clear that this distribution was similar in the two species. Consequently, studies on the chick duodenum will only be mentioned when differences between the two species were encountered.

Since the physiological movement of calcium is from the lumen across the epithelium and into the circulatory system within the lamina propria, only this region was investigated. This section is therefore conveniently divided into studies of the individual cellular components: the goblet cells, the absorptive cells, and regions of the lamina propria.

GOBLET CELLS: An unexpected observation in this study was the frequent presence of calcium within goblet cells. This distribution was observed regardless of intestinal region. In the rat duodenum in Fig. 1 *b*, calcium is concentrated in the upper right quadrant, and by referring to the sample current picture is found in the area of mucin accumulation within the goblet cell. Fig. 2 shows a similar calcium distribution in rat ileum; an identical distribution is found in jejunum. With the number of goblet cells increasing from duodenum to ileum (26), there is a corresponding increase in the number of goblet cell calcium

localizations. These localizations occur within goblet cells along the entire length of the intestinal villus, and are also frequently observed within goblet cells of the intestinal crypts.

Within the goblet cell, calcium localizations are found only in regions of mucin production (goblet cell neck) or accumulation (theca), thus indicating an association between localizations and mucin. Fig. 3 shows a portion of a goblet cell which contains throughout a diffuse distribution of calcium. This diffuse distribution is more pronounced than that normally observed. Also apparent are the more common discrete localizations which are compatible with the structure and size of mucigen droplets as observed in the electron microscope (Fig. 22).

Similar calcium localizations were observed in goblet cells from the duodenum of mature (approximately 1-yr old) rats, in rats deprived of food and water 24 h before sacrifice, and in chicks. However, although approximately 90% of goblet cells in the rat contained calcium localizations, far fewer goblet cells in the chick contained calcium. In addition, whereas the goblet cell mucin appears nearly white in tone in the rat, in chick duodenum the mucin appeared dark and could not be distinguished from the tone of clear plastic. This indicates a significant difference in the average atomic number of the embedded mucin from the two species.

The association between goblet cell mucin and calcium is not affected when goblet cells extrude the accumulated mucin. The mucus being extruded in Fig. 4 is still in globular form and calcium remains associated with this mucus. Fig. 5 shows a goblet cell that has extruded most of its mucus, which then forms a layer over the surface of the epithelial cells. The calcium is still associated with the extruded mucus, and still localized. Calcium therefore appears to be bound to mucin within goblet cells and to remain associated with the mucus outside the cells.

ABSORPTIVE CELLS: In the duodenum of fasted rats, no calcium signal above background was detected in X-ray images of absorptive cells. Usually no signal above background was detected in absorptive cells of animals fed *ad libitum*. This can be seen in Figs. 1 and 2, in which detectable calcium is associated only with goblet cell mucin. Infrequently, however, discrete localizations similar to those occurring in goblet cells were observed in absorptive cells of animals fed *ad libitum*. Fig. 6 shows three calcium localizations, one within a

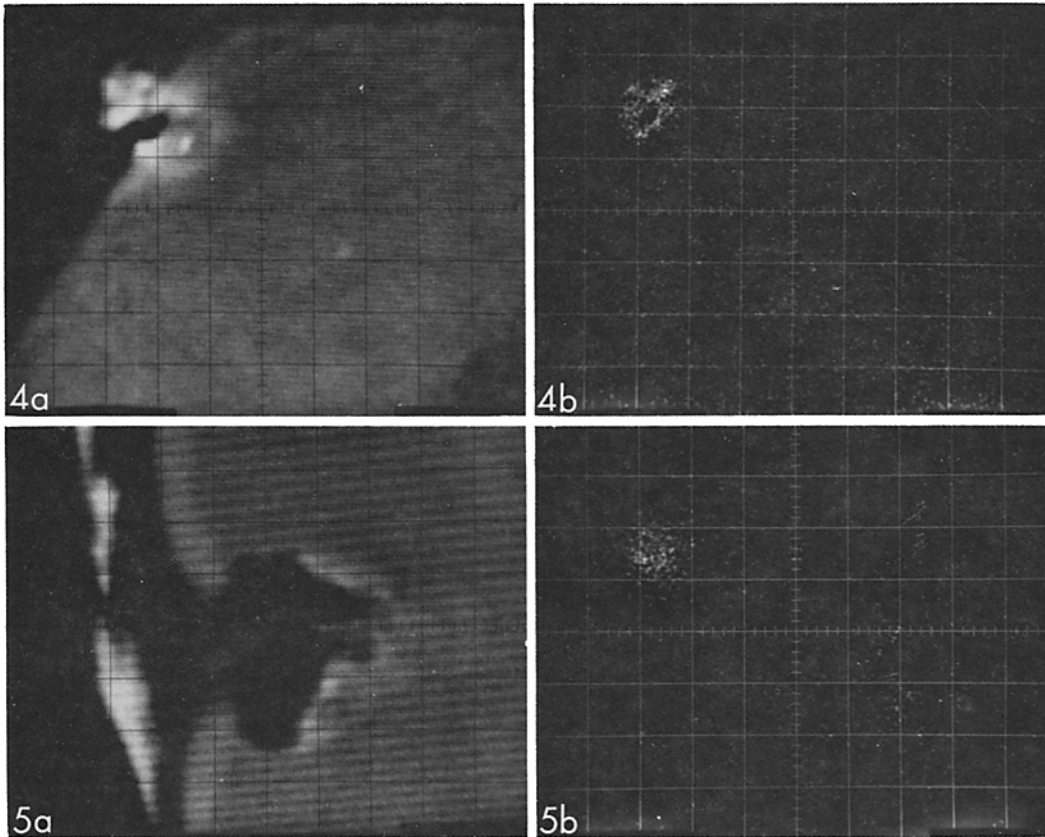


FIGURE 4 A transversely cut section of epithelium from rat duodenum. A goblet cell can be seen extruding its accumulated mucin; substantial amounts of calcium remain associated with the mucus. The scale is $3 \mu\text{m}/\text{SGS}$.

FIGURE 5 This goblet cell from rat duodenum has extruded most of its mucus. The calcium is still localized and associated with the extruded mucus. The scale is $2 \mu\text{m}/\text{SGS}$.

goblet cell. The remaining localizations are shown at higher magnification in Figs. 7 and 8. In Fig. 7 the calcium localization appears relatively diffuse and occupies a large area encompassing portions of the brush border, lateral cell border, and cell cytoplasm. It is likely, however, that the calcium X-ray image is somewhat larger than the actual calcium source; the actual size of a localization cannot be determined from just an X-ray image (10). In Fig. 8 the calcium localization appears in the cell cytoplasm separate from the lateral cell membrane; in addition, a smaller localization further into the tissue is now visible at this magnification. It is important to note that there is no evidence of a calcium concentration gradient across the epithelial cells. Line scans and point analyses were performed to determine whether

calcium concentration gradients extended from the luminal to the basal surface of absorptive cells. No evidence of such a gradient could be found. When calcium was observed, it was not in the form of a continuous gradient but appeared as discrete localizations.

LAMINA PROPRIA: Occasional discrete localizations were observed in the lamina propria throughout the small intestine. Fig. 9 shows a calcium localization just beneath the epithelium near what appears to be a capillary. Fig. 10 shows a calcium localization near a lacteal or capillary. The calcium localizations in the lamina propria frequently gave calcium X-ray counting rates 2-5 times that encountered in epithelial cells. Localizations within the lamina propria were encountered more frequently in chick than in rat.

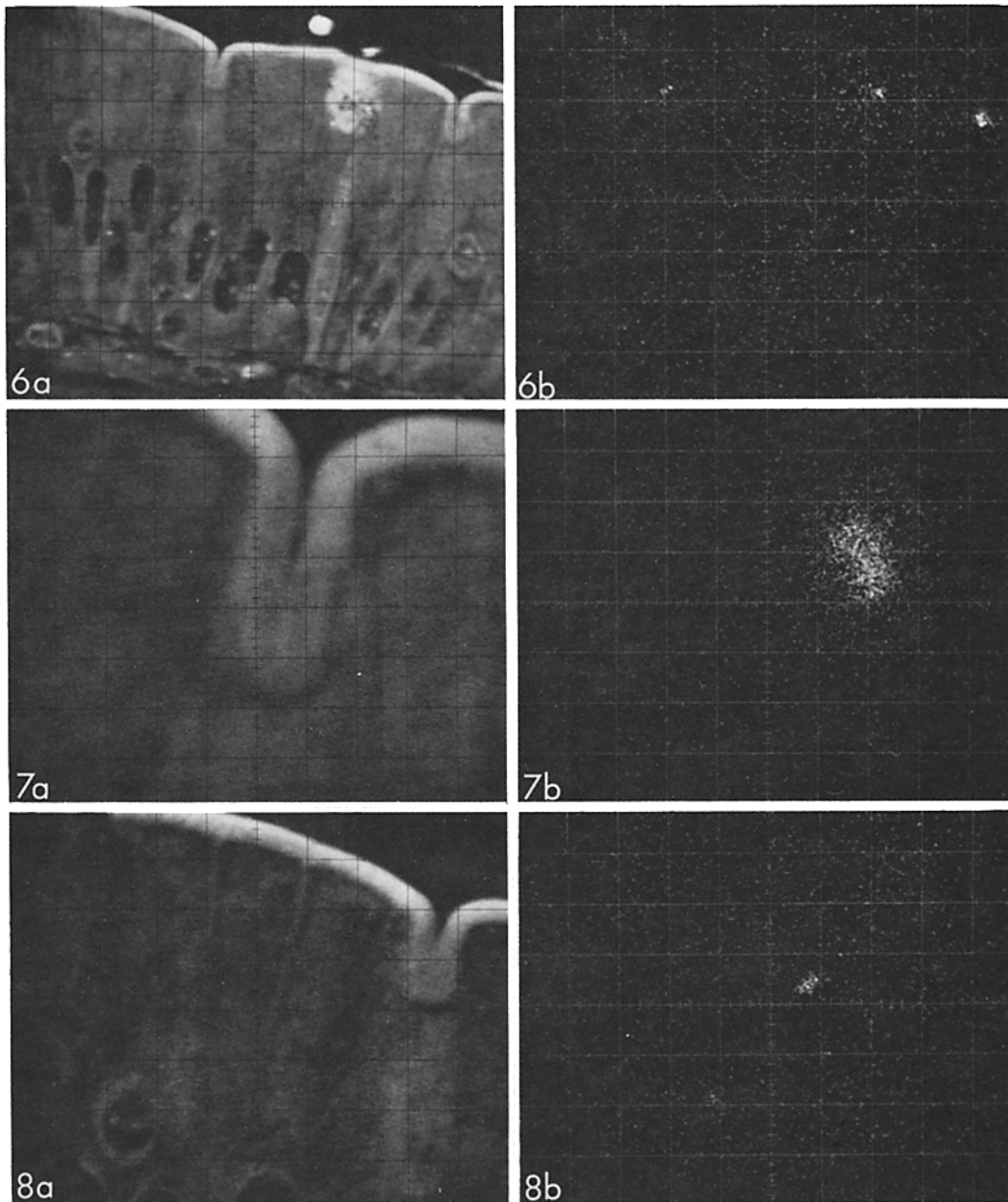


FIGURE 6 A portion of a villus from rat duodenum. Calcium localizations can be seen within the goblet cell and the absorptive cells. The animal was fed ad libitum. The scale is $8 \mu\text{m}/\text{SGS}$.

FIGURE 7 A magnification of Fig. 6 showing a calcium localization near the brush border within the epithelium. The scale is $1 \mu\text{m}/\text{SGS}$.

FIGURE 8 Magnification of Fig. 6 showing a calcium localization within an absorptive cell. Although it is not immediately apparent, higher magnification indicates that this localization is not associated with the lateral intercellular space. This image reveals in addition the presence of another calcium localization which has penetrated further into the epithelium. The scale is $3 \mu\text{m}/\text{SGS}$.

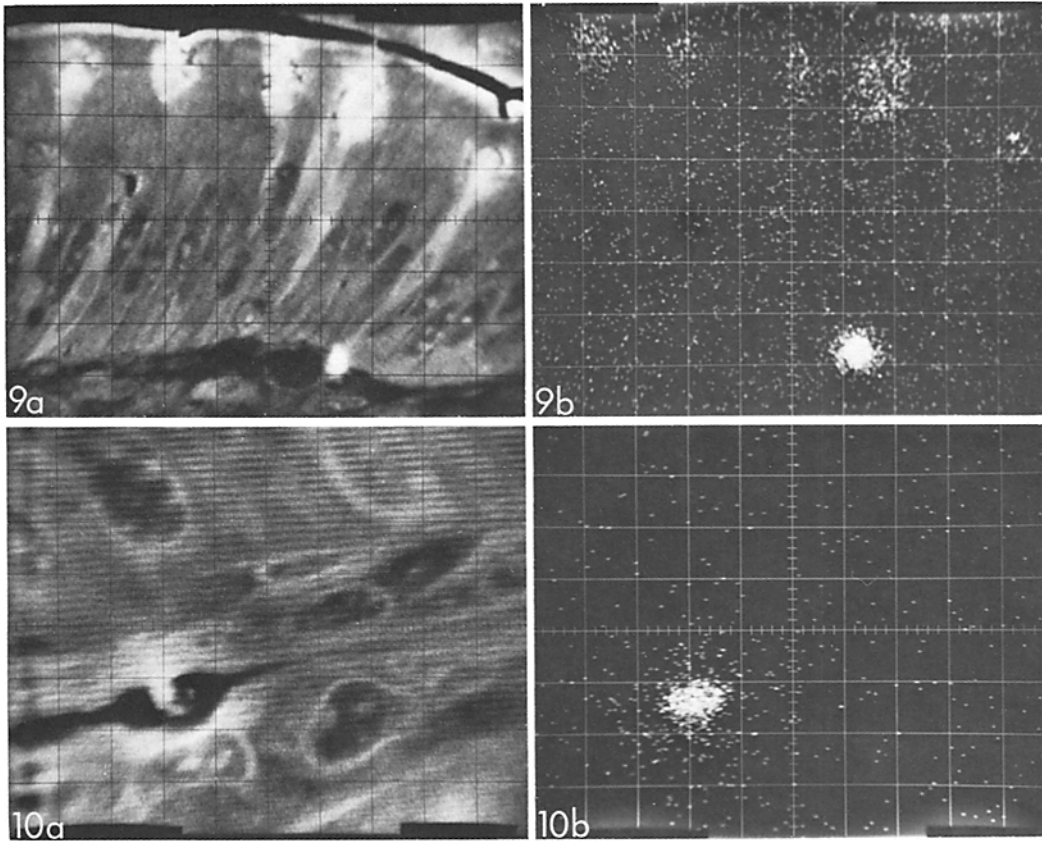


FIGURE 9 A portion of a villus from rat duodenum showing a large calcium localization at the base of the epithelium in the lamina propria near what appears to be a capillary. The scale is $8 \mu\text{m}/\text{SGS}$.

FIGURE 10 The lamina propria from this rat ileum is a diagonal band below the center of the image. The lower right corner is the base of several epithelial cells. An absorptive cell nucleus can be seen just to the right of the upper left corner. A calcium localization is within the lamina propria associated with a lacteal or capillary. The scale is $3 \mu\text{m}/\text{SGS}$.

II. Calcium Distribution Under *In Vitro* Transport Conditions

Having established the normal *in vivo* calcium distribution, incubation experiments were performed in order to determine the form and path of calcium translocation. Tracer studies were used to demonstrate calcium uptake by the duodenum with the incubation conditions employed. The time course of ^{45}Ca uptake is presented in Chart 1. In order to eliminate the surface exchange factor, the procedure utilized by Martin and DeLuca (25) was followed in which tracer uptake after 1 min was designated as 0 and subtracted from the 5- and 15-min incubations. The results are similar to

those obtained by Martin and DeLuca with this technique (25) (Fig. 5).

Tissue from the control (i.e., no incubation) was prepared in the same manner as that previously described in part I and exhibited the same calcium distribution. Tissue incubated for 5 min exhibited the calcium distribution shown in Fig. 11, characterized by discrete calcium localizations within the absorptive epithelium near the luminal surface. The localizations have penetrated almost equally into the epithelium; excepting the lobular indentations, they present a nearly linear array with the epithelial surface. At higher magnification, most of these localizations are near the lateral cell membranes, as in Fig. 12, where the localization is at the level of the junctional complex. Occasional

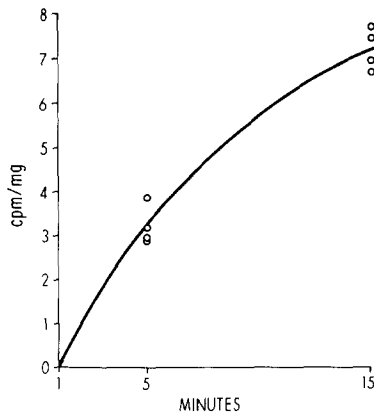


CHART 1 ^{45}Ca uptake by rat duodenum according to the procedures of Martin and DeLuca (25). The origin represents the amount of ^{45}Ca taken up by the tissue after 1 min of incubation, in order to compensate for surface binding. The abscissa represents counts per minute per milligram of tissue.

localizations appeared within cells removed from these lateral membranes, as seen in Fig. 13.

Tissue incubated for 15 min generally no longer had localizations near the apical border. As seen in Fig. 14, the localizations have penetrated further into the tissue while roughly maintaining their linear distribution parallel to the epithelial surface. Most of these localizations are near a lateral cell border, although others appear to be at the cell center in a supranuclear position. An example of this latter supranuclear distribution is seen at higher magnification in Fig. 15.

Although the calcium distribution represented by Fig. 14 is commonly encountered after 15 min of incubation, localizations are also encountered near the apical border, as seen in the adjacent villus in Fig. 14, and associated with the intercellular space at or below the nucleus, as in Fig. 16. Less frequently, calcium localizations are found simultaneously near the nuclei and near the apical border in the same villus area. Although local regions of a villus appear to have the same calcium distribution, this distribution varies from villus to villus and may vary even on the same villus. Consequently, not every cell appears to be equally involved in calcium transport.

In the chick, calcium localizations appear to move more quickly across the cells. After 15 min of incubation, localizations were frequently found in a linear array within the lamina propria or near the epithelial cell base.

From nuclei to the lamina propria, calcium localizations were only observed near the intercellular space, as represented by Fig. 16. Localizations were not observed within nuclei or in the cell cytoplasm below the nuclei.

Since sections for EPMA were 1- or 2- μm thick, it is possible that a section could include portions of two cells, and thus include the intercellular space between the cells. Since a sample current image is a two-dimensional projection of the section, a calcium localization in the intercellular space between the two cells might appear in the sample current image to be in the center of one cell. To exclude this possibility, 1- μm thick sections were cut diagonally to the long axis of the columnar cells. In a section of this type, only a very small portion of the intercellular space will be included, and these intercellular spaces are so clearly defined that chances for misinterpretation of the calcium location are almost entirely eliminated.

Fig. 17, from a 5-min incubation, shows an example of this kind of section. Transverse sections from tissue incubated for 15 min are similar to Fig. 17, except that calcium localizations have penetrated further into the epithelium and are no longer only associated with the apical border. Although most localizations were near lateral membranes, occasionally calcium localizations were clearly shown to be within the cell cytoplasm in a supranuclear position, as in Fig. 18.

Incubation did not appear to increase the amount of calcium associated with goblet cell localizations. The mean X-ray intensity from localizations in the *in vivo* preparations was 65.0 ± 9.9 (SEM) cpm, and from incubated preparations was 73.5 ± 12.5 (SEM) cpm. There is no statistically significant difference between these values. As shown in Table I, however, there may be a greater amount of calcium associated with absorptive cell calcium localizations than is found in goblet cells; the two values for normal animals are statistically different ($P < 0.05$).

With a static electron beam positioned on a calcium localization, the entire X-ray wavelength spectrum was scanned to determine what anions might be associated with the calcium localizations. Particular attention was given to phosphorus, chlorine, sulfur, and arsenic (the anion for the fixative buffer contains arsenic). However, no signal above background (measured in cell cytoplasm removed from calcium localizations) could

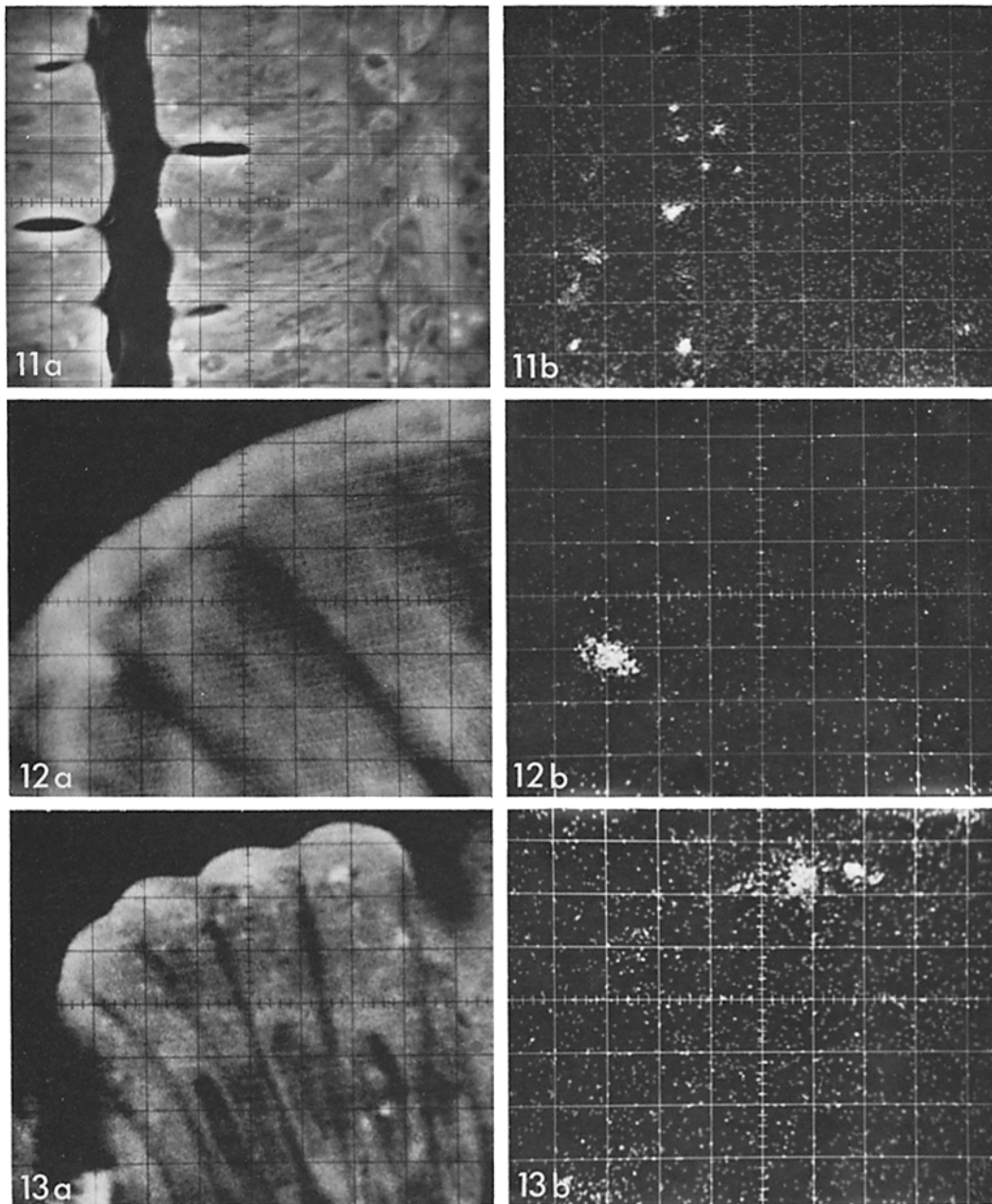


FIGURE 11 A portion of two villi from rat duodenum incubated for 5 min in a solution containing 2 mM Ca. Calcium localizations are found near the apical surface within the epithelium and predominantly near the lateral cell borders. The scale is 10 $\mu\text{m}/\text{SGS}$.

FIGURE 12 The apical surface of several absorptive cells from rat duodenum incubated for 5 min in a solution containing 2 mM Ca. A calcium localization is near the lateral cell border at the level of the junctional complex. The scale is 1.5 $\mu\text{m}/\text{SGS}$.

FIGURE 13 A portion of a villus from rat duodenum incubated for 5 min. The calcium localizations appear to be intracellular just beneath the terminal web. The scale is 4 $\mu\text{m}/\text{SGS}$.

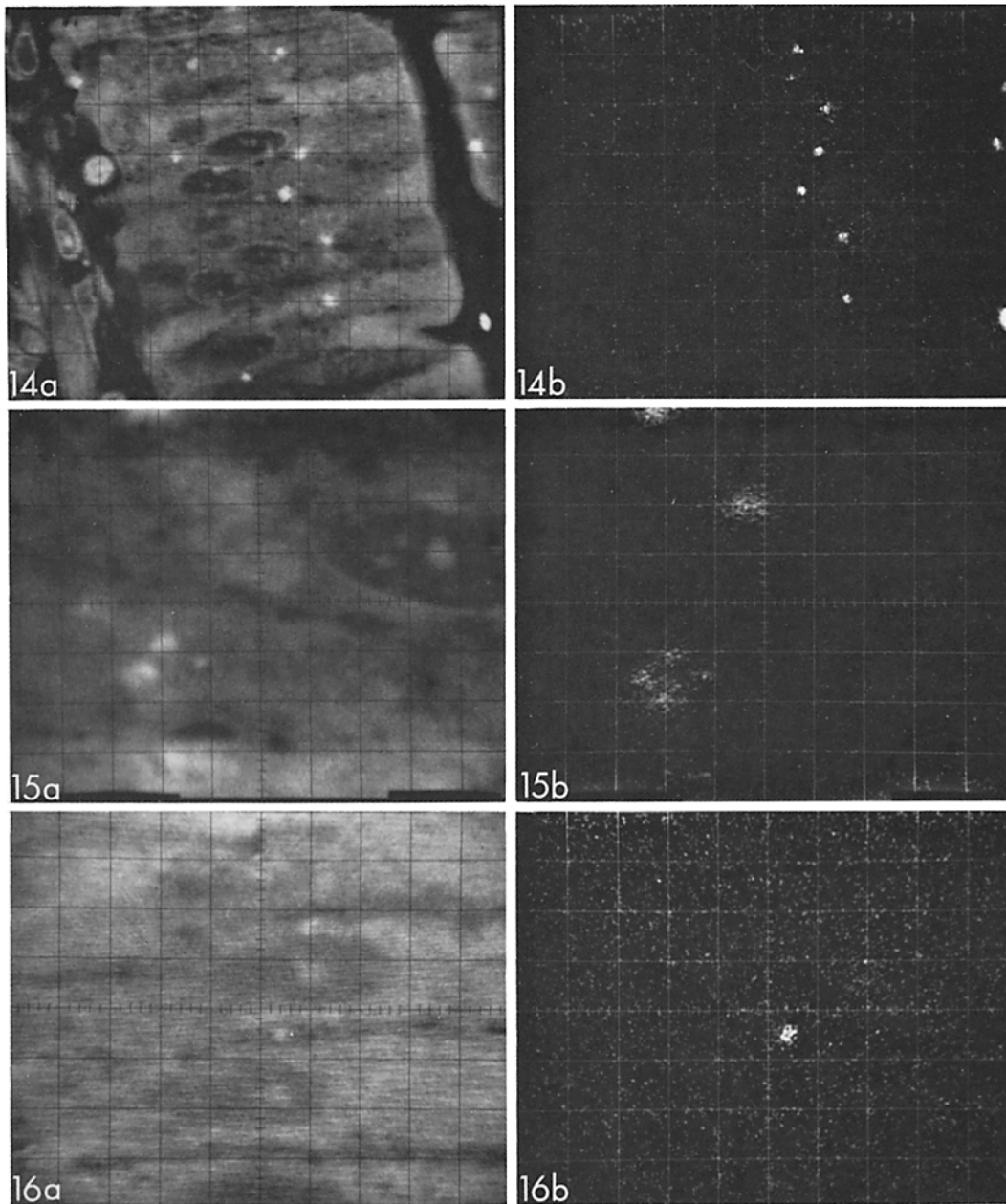


FIGURE 14 A portion of two villi from rat duodenum incubated for 15 min in a solution containing 2 mM Ca. Of the seven central localizations, the inner five are near lateral cell borders, whereas the outer two appear to be intracellular in a supranuclear position. The small portion of the adjacent villus at the right side of the image shows two localizations still near the apical border. The scale is $7 \mu\text{m}/\text{SGS}$.

FIGURE 15 A central area of several absorptive cells from rat duodenum incubated for 15 min in a solution containing 2 mM Ca. The calcium localizations appear to be intracellular in a supranuclear position. The apical surface lies to the left. The scale is $2 \mu\text{m}/\text{SGS}$.

FIGURE 16 A central area of several absorptive cells from the chick duodenum incubated for 15 min in a solution containing 2 mM Ca. A calcium localization is at the level of the nucleus associated with the region of the intercellular space. The apical surface lies to the left. The scale is $2 \mu\text{m}/\text{SGS}$.

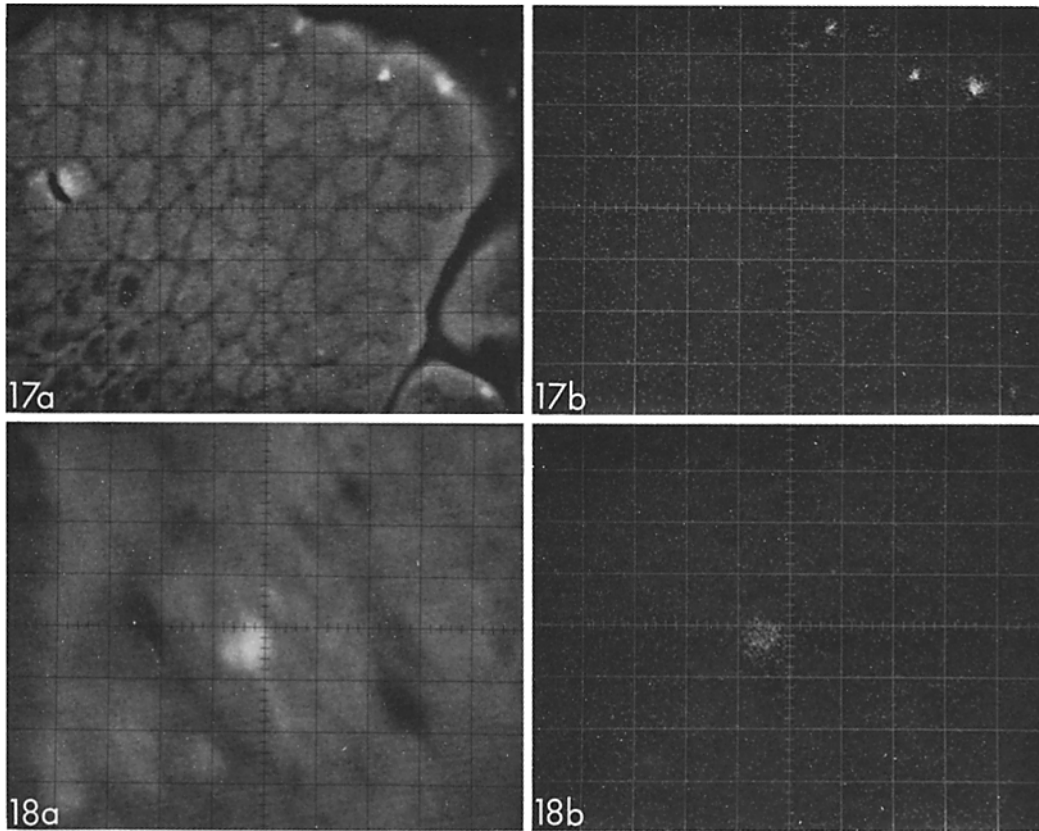


FIGURE 17 A transverse section of a villus from rat duodenum after 5 min of incubation. The localizations appear to be associated with the intercellular space near the junctional complex. Cross sections of cells containing nuclei can be seen at the lower left corner. The scale is $8 \mu\text{m}/\text{SGS}$.

FIGURE 18 A transverse section from rat duodenum after 15 min of incubation. One complete cell taken in cross section at the center of the image is shown surrounded by portions of five or six other cells. A calcium localization clearly removed from the lateral borders is shown within the complete cell. The scale is $1 \mu\text{m}/\text{SGS}$.

be detected. The calcium in a localization was, therefore, associated with oxalate or other organic anion.

III. Calcium Distribution in Vitamin D-Deficient and Replete Animals

If the discrete localizations described above represent calcium being transported, their occurrence and distribution should be consistent with experimental alteration of the parameters of transport. Vitamin D is an agent with well-documented effects on calcium transport (42, 45). Depleting an animal of vitamin D will suppress calcium transport, and repleting such an animal with vitamin D will restore and enhance the transport ability. Thus

EPMA of D-deficient and replete animals would distinguish the changes in cellular calcium that are associated with the calcium transport process.

In unincubated controls from duodenum of vitamin D-deficient hypocalcemic rats, calcium is observed only in the form of localizations within goblet cells and within the extruded goblet cell mucus (Fig. 19). Absorptive cells from D-deficient rats are devoid of calcium localizations. This calcium distribution was unchanged after *in vitro* incubation; in particular, no calcium localizations within absorptive cells were observed.

The occurrence of calcium localizations within goblet cells of D-deficient hypocalcemic rats was an unexpected observation. The amount of calcium in these goblet cell localizations does not appear to

TABLE I
X-Ray Intensities from Calcium Localizations of Normal, Vitamin D-Deficient, and Vitamin D-Replete Rats

Normal		Hypocalcemic		D-Replete	
Cell	Goblet	Cell	Goblet	Cell	Goblet
N = 59	N = 41	None	N = 25	N = 34	N = 9
\bar{X} = 87.6	\bar{X} = 69.2	None	\bar{X} = 70.7	\bar{X} = 78.0	\bar{X} = 121.2
SE = 5.3	SE = 7.4	None	SE = 9.2	SE = 6.4	SE = 22.6

N, number of localizations; SE, standard error; \bar{X} , mean uncorrected Ca K_{α} X-ray counts per second.

be different from that in normal animals (Table I), and did not appear to be affected by incubation in a calcium solution.

In unincubated controls from D-replete rats, calcium localizations were present in goblet cells and were commonly observed within absorptive cells (Figs. 20, 21). In both control and incubated tissue the calcium localizations had the same morphological distribution and appeared the same as those observed in normal tissue. The localizations within goblet cells of D-replete rats contained large amounts of calcium (Table I). Incubation had no apparent effect on these calcium localizations; however, at times an extremely large amount of calcium was observed associated with extruded extracellular mucus in incubated tissue.

As before, results will be presented primarily from the rat. Difficulties were encountered in maintaining a suitable environment for raising rachitic chicks, producing chicks with a range of hypocalcemic serum calcium levels from clearly rachitic to almost normal. This meant, however, that it was possible to compare the electron probe results from animals with a range of serum calcium levels. In this way additional clarification of the effects of vitamin D on the calcium transport process was obtained.

Investigation of the effects of vitamin D on the chick indicated that calcium localizations in these animals varied roughly as did the serum calcium levels. When serum calcium levels were high (approximately 8 mg/100 ml), there was no obvious distinction in the number of localizations between rachitic and normal states, nor did vitamin D repletion have an obvious effect. However, small changes in the frequency of occurrence of localizations would be difficult to detect. In animals with low serum calcium levels, differences between rachitic, normal, and D-replete states were more apparent, although never as clearly defined as that observed with the rat. It was quite

apparent, however, that in animals with low serum calcium levels, the number of calcium localizations were drastically decreased. When localizations were observed, they exhibited the same distribution as that found in normal animals.

Calcium localizations in vitamin D-replete animals were analyzed for the presence of other elements. As in normal animals, calcium was associated only with oxalate or other organic compounds.

The inability of the electron microprobe to detect low levels of calcium when operated in the scanning mode has previously been mentioned. To determine whether this insensitivity was permitting the visualization of one calcium transport process, i.e., calcium localization, but obscuring another (perhaps diffuse) process, such as an increased but low level transport through every cell, point counts were taken on the apical cytoplasm of both control and incubated rat absorptive cells from normal, vitamin D-deficient, and vitamin D-replete animals. In nearly every instance, the calcium X-ray counts on the tissue not only exceeded background (measured at the same site with the spectrometer tuned off-peak), but also exceeded background plus one standard deviation (\sqrt{N}), thus indicating tissue calcium was indeed being detected. An example of the X-ray counts before correction (randomly selected) is presented in Table II along with a control measurement taken on pure plastic adjacent to embedded tissue.

The BASIC-corrected counts from the cytoplasm of various cells are presented in Table III. Results at these extremely small counting rates are subject to large errors; however, it would appear that incubation did not increase the cell calcium concentration in vitamin D-deficient animals. It is also clear that the cell calcium concentration in normal and vitamin D-replete animals was significantly elevated by incubation. This suggests that there may be another calcium

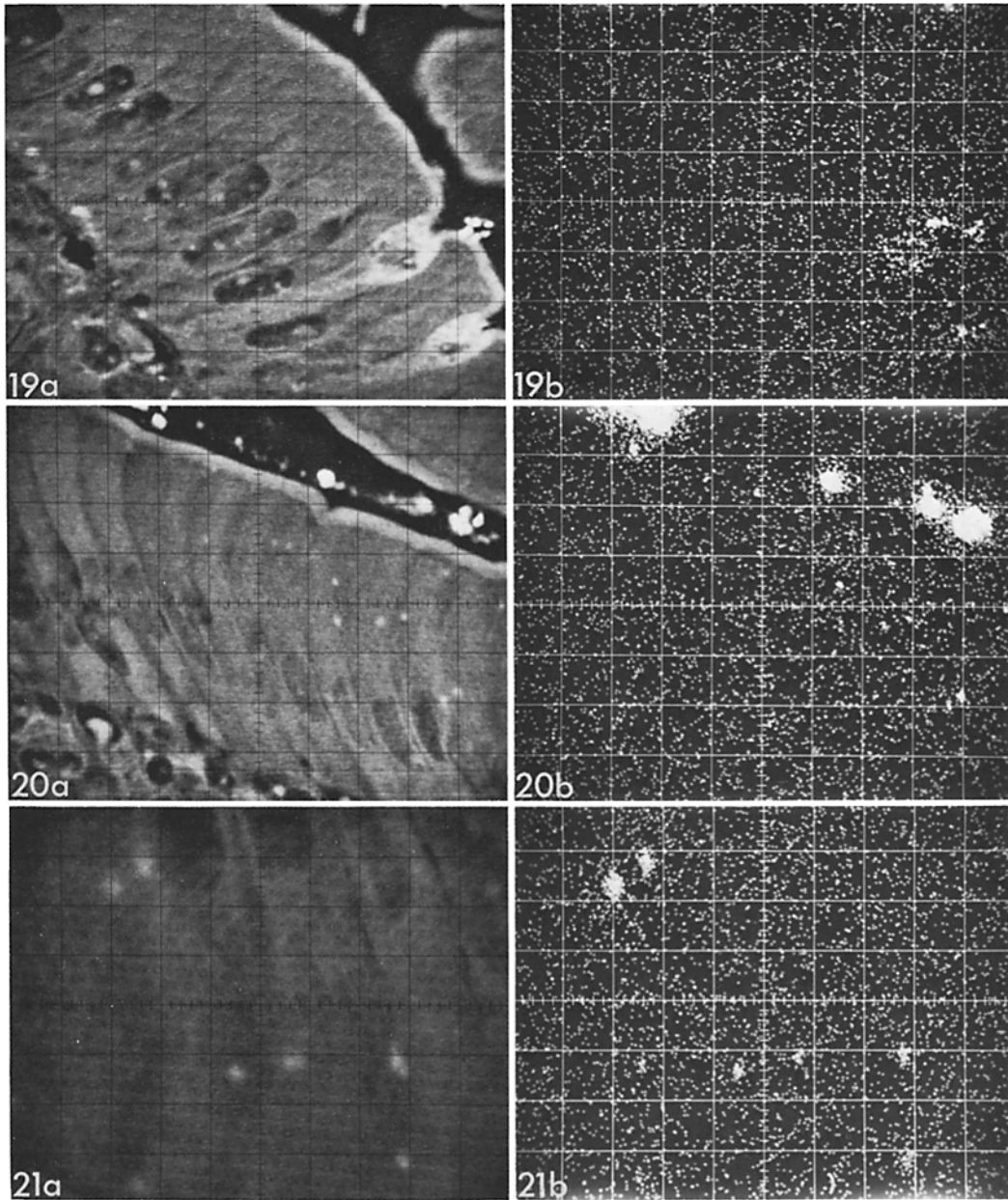


FIGURE 19 A portion of a villus from the duodenum of a vitamin D-deficient rat. Calcium localizations are observed both within goblet cells and associated with the extracellular goblet cell mucus. No localizations were observed within the absorptive cells. The scale is $8 \mu\text{m}/\text{SGS}$.

FIGURE 20 A portion of a villus from the duodenum of a vitamin D-replete rat. Calcium localizations are within the absorptive cells and associated with extracellular goblet cell mucus. The scale is $8 \mu\text{m}/\text{SGS}$.

FIGURE 21 A magnification of Fig. 20 showing the calcium localizations in more detail. The apical surface is toward the bottom of the image. The scale is $3 \mu\text{m}/\text{SGS}$.

transport process which is not represented by the discrete localizations.

IV. Analysis of the Sample Preparation Procedure

The significance of observations in parts I and II depends on the demonstration that discrete localizations are not artifacts of the sample preparation procedure. A study describing the effects of this sample preparation procedure on another calcium transporting tissue, the embryonic chick chorioallantoic membrane, indicated that the normal distribution of calcium was preserved in this tissue (10, 11), and previous work had shown that oxalate can be used to preserve the distribution of calcium in striated muscle (13, 29). The results of part III indicate that these localizations are consistent with the known physiological actions of vitamin D. This result, however, is not a sufficient reason to dismiss the possibility that artifacts have been created; effects of the sample preparation procedure must be investigated. Elimination of likely sources of calcium redistribution can be used to further support the claim that the preparative procedure maintains the normal distribution of calcium. For instance, since the membrane systems of a cell represent phase boundaries, any indication that cell membranes have been destroyed during sample processing increases the probability that artifactual translocations have occurred. Figs. 22 and 23 show electron micrographs demonstrating that the fine structural characteristics and membrane systems of the cells are well preserved with this preparative technique. A more complete discussion of sources of calcium redistribution has been given (10).

Essential to the preparative technique is a

TABLE II

Uncorrected X-Ray Counts Taken on and off the Ca K_α X-Ray Peak with Electron Beam on Pure Plastic and on Plastic-Embedded Rat Absorptive Cell Cytoplasm

	On peak	Off peak
Plastic	522	555
Tissue	663	617
	686	625
	693	594
	705	648

5-min counts.

TABLE III

BASIC Corrected Ca Concentrations in Atomic Percent $\times 10^2$ from the Apical Cytoplasm of Incubated and Nonincubated Absorptive Cells of Normal, Vitamin D-Deficient, and Vitamin D-Replete Rats

Experiment	Concentration in atomic percent $\times 10^2$		
	Control	Incubated	
R ₁	2.62 \pm 0.70	2.15 \pm 0.89	NS
R ₂	1.39 \pm 1.24	2.65 \pm 2.57	NS
R ₃	1.52 \pm 0.73	1.93 \pm 2.72	NS
Normal	1.62 \pm 1.17	5.92 \pm 2.72	P* < 0.0025
D ₁	2.34 \pm 0.31	4.55 \pm 2.11	P* < 0.0125
D ₂	1.32 \pm 0.32	5.15 \pm 4.69	P* < 0.0500

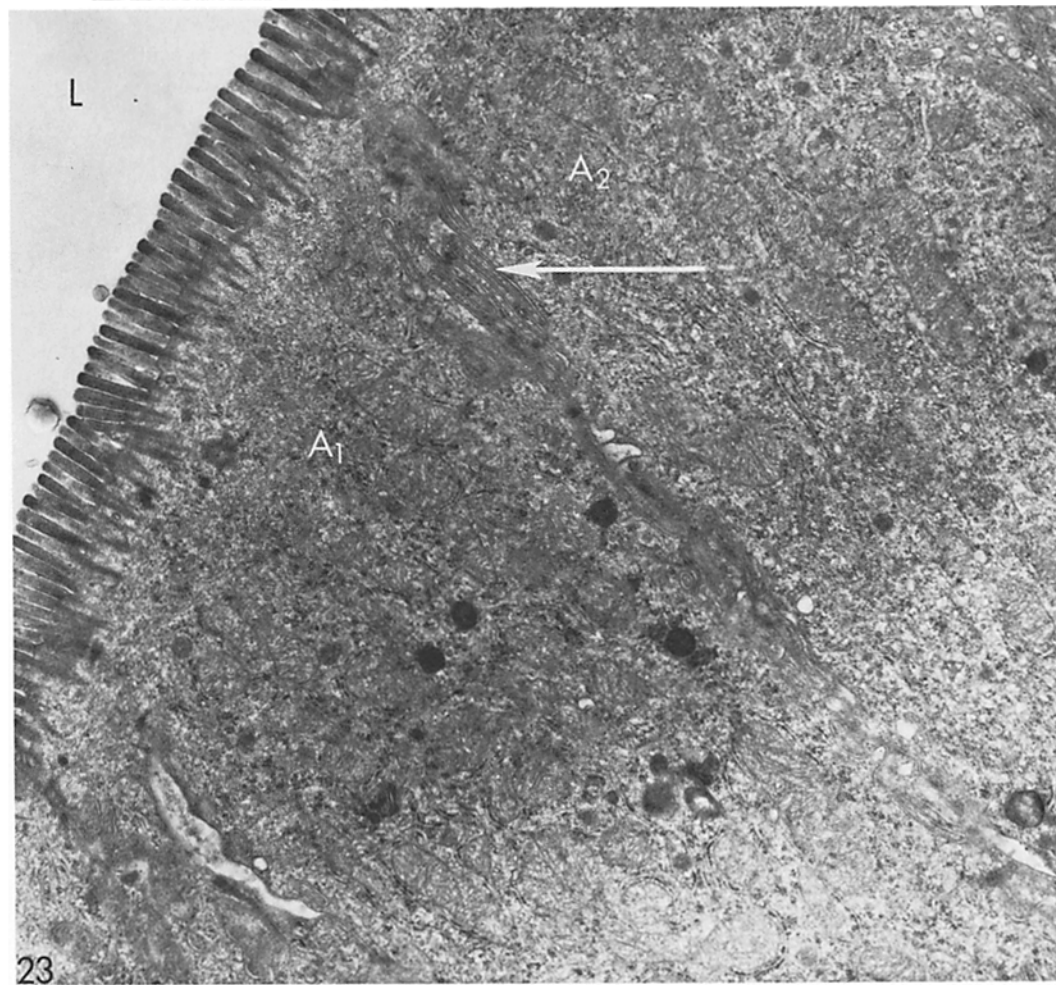
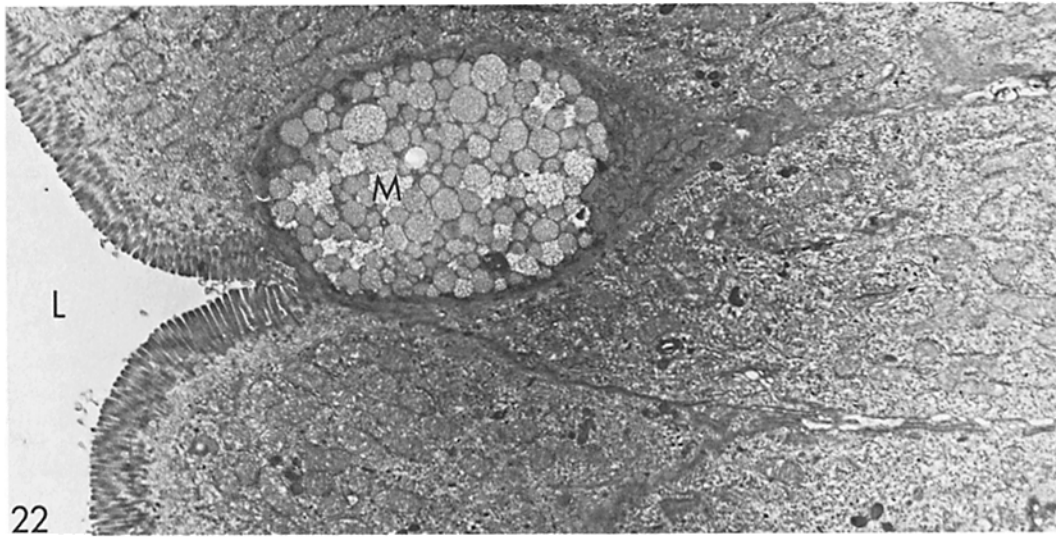
The values given are the means \pm the standard deviation.
* According to Student's *t* test.

calcium-precipitating agent (oxalate) and a fast-acting fixative. When oxalate is omitted from the sample preparation fluids, calcium is no longer detected in the tissue, as seen in Fig. 24. This demonstrates that if calcium is allowed freely to diffuse, as in Fig. 24, it is not maintained at specific sites. Consequently, the calcium distributions reported in parts I, II, and III are the result of immobilizing calcium with the precipitating agent oxalate, and do not arise from the free diffusion of calcium.

Acrolein is the fastest and most effective fixative (16).¹ Osmium tetroxide is a slow-diffusing fixative which has been reported to increase tissue permeability to calcium (28). If osmium tetroxide (plus oxalate) is used instead of acrolein (plus oxalate), an obvious redistribution artifact is observed. This distribution, as seen in Fig. 25, is characterized by extremely large amounts of calcium located extracellularly between villi. Except for infrequent small localizations of calcium within goblet cells, no distribution above background was observed within the tissue. Therefore, the use of a technique known to redistribute calcium produces a quite different distribution from that described earlier.

If the use of aqueous fluids in the preparative technique artificially creates localizations from an originally nonlocalized (i.e., diffuse) distribution, then the reapplication of an aqueous fluid to the localizations should not reverse the process and recreate the original diffuse distribution. Fig. 26 *a* is a sample current image from rat duodenum; the

¹ W. Aldridge. 1972. Personal communication.



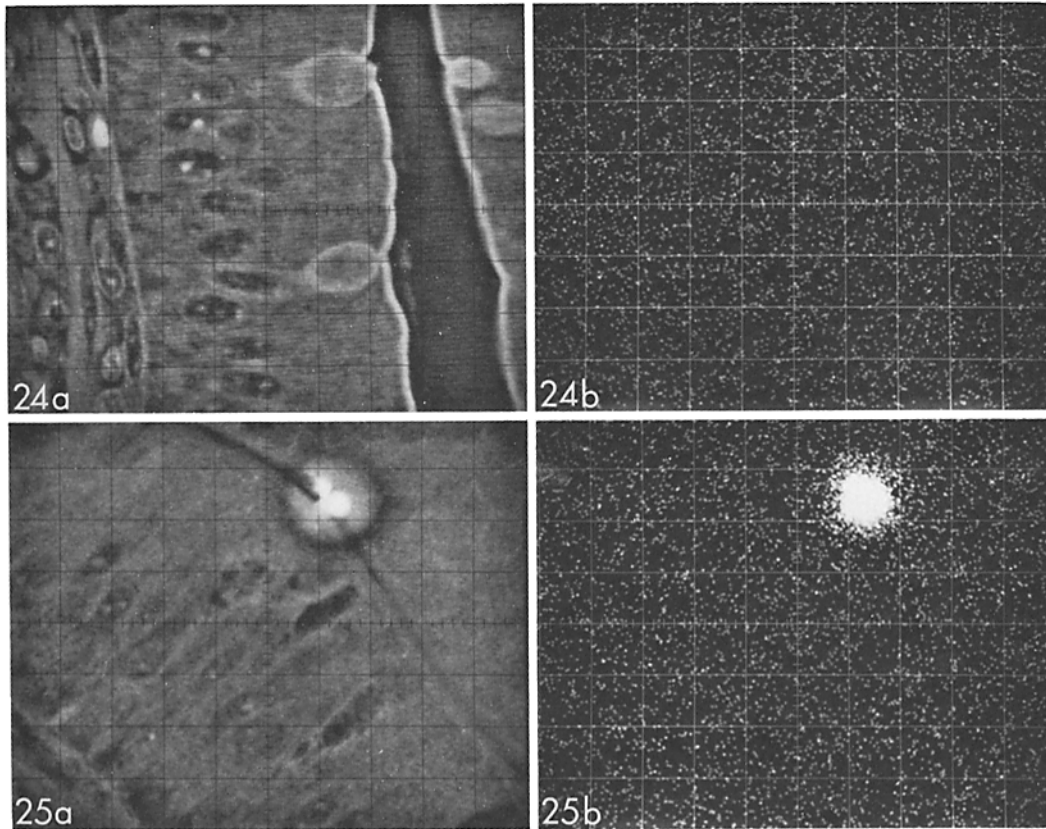


FIGURE 24 A portion of a villus from the rat duodenum fixed without oxalate in the sample processing fluids. Calcium is not retained by the tissue. The scale is $8 \mu\text{m}/\text{SGS}$.

FIGURE 25 A portion of two villi from chick duodenum fixed with osmium plus oxalate instead of acrolein. The lamina propria of one villus can be seen in the lower left corner. The other villus is in the upper right corner and was cut in transverse section. A large amount of calcium is localized extracellularly between the villi and would appear to be a redistribution artifact. The scale is $8 \mu\text{m}/\text{SGS}$.

corresponding calcium X-ray image is adjacent (Fig. 26 b).

A drop of distilled, deionized water was placed over the section and evaporated to dryness on a

60°C hotplate, the evaporation taking approximately 1 min. The resulting calcium distribution is seen in Fig. 26 c. Many of the localizations are removed. The remaining localizations are not

FIGURE 22 An electron micrograph of rat duodenum. The tissue was processed as described in the text. Tissue morphology appears very similar to that of tissue prepared by more conventional techniques. The white area to the left is the intestinal lumen (*L*). Portions of several absorptive cells and a goblet cell are shown. The luminal end of the goblet cell is filled with spherical bags of mucin, or mucigen droplets (*M*). $\times 3,650$.

FIGURE 23 An electron micrograph of the apical portion of several absorptive cells from rat duodenum. The fine structural characteristics and membrane systems of the cells are shown to be well preserved by the preparative technique. The intestinal lumen (*L*) is to the left. Two absorptive cells (*A*₁ and *A*₂) are separated by intact interdigitated lateral membranes (arrow). Endoplasmic reticulum and mitochondria also have a normal appearance. The mitochondria do not contain dense deposits. $\times 10,600$.

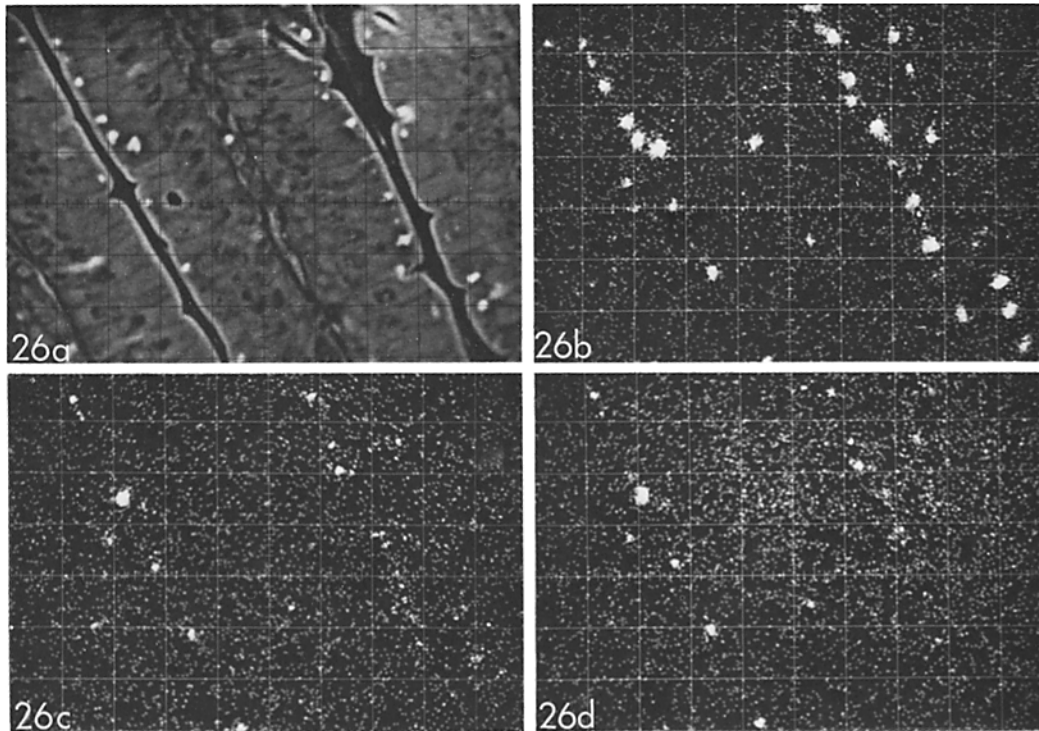


FIGURE 26 The effect of distilled water and 5 mM CaCl_2 on calcium localizations within epithelial tissue. (a) Sample current image of portions of three villi from rat duodenum; (b) corresponding Ca K X-ray image; (c) the calcium distribution after exposure to water; (d) the calcium distribution after exposure to 5 mM CaCl_2 . Since it was necessary to remove the sample from the microprobe for each operation (c) and (d), specimen reorientation did occur. Due to the inability exactly to align the sample as it was originally, the calcium localizations are not found in exactly the same place with reference to the grid bars. An inspection at high magnification before and after each operation verified that there was no redistribution of the localizations. The scale for all images is $19 \mu\text{m}/\text{SGS}$. All X-ray integrations were for 30 min.

enhanced nor are previous localizations redistributed; calcium lost from localizations does not reaggregate and is instead diffusely and nonspecifically bound, as seen by the increased background. A drop of 5 mM CaCl_2 was then placed over the same section, maintained over low heat (30°C) for 10 min, and removed with filter paper. The resulting calcium distribution is seen in Fig. 26 d. The existing localizations are not enhanced, nor are new localizations created. Instead, there is an increased diffuse distribution. Therefore, conditions that might have caused the artifactual formation of localizations instead cause a redistribution in the opposite direction to form diffuse distributions.

As previously reported (11) a homogeneous calcium distribution was prepared by dissolving calcium chloride in 10% albumin and processing

the mixture in the identical manner as the analyzed tissue. Microprobe analysis revealed a homogeneous calcium distribution, as seen in Fig. 27. This experiment demonstrates that the specimen preparation procedure does not produce localizations; a diffuse distribution, if present, is maintained. This result strongly indicates that discrete localizations are not artifactual. This conclusion is supported by Fig. 3 where both distributions, diffuse and localized, are maintained.

Finally, the distinct patterns of the localizations themselves argue against the occurrence of redistribution. The localizations are not random as one might expect in a redistribution artifact.

DISCUSSION

The occurrence of discrete calcium localizations is neither an unexpected nor unpredicted result for

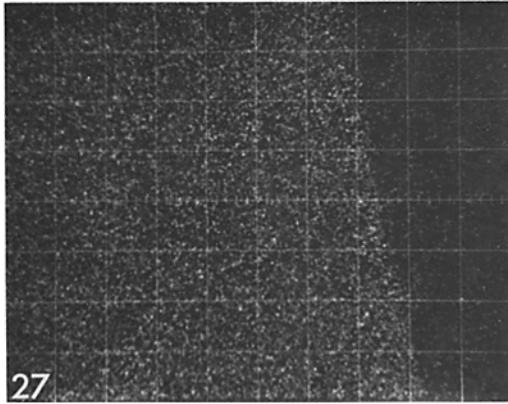


FIGURE 27 Calcium X-ray image of a section of an albumin and CaCl_2 mixture processed as described for intestinal tissue. The left portion of the image corresponds to the albumin and the right portion is the embedding plastic. It can be seen that the original homogeneous distribution of calcium is maintained by the preparative procedure. The calcium X-ray intensity for this $1\text{-}\mu\text{m}$ thick section was 12 counts/s and the scale is $8\ \mu\text{m}/\text{SGS}$.

two reasons. First, a similar calcium distribution was encountered in certain cells of the calcium-transporting chick chorioallantoic membrane (9, 10, 11). Second, it is generally accepted that cells maintain a constant, low (about $10^{-6}\ \text{M}$) cytoplasmic calcium activity (4). A level of ionic calcium much above this value is deleterious to the normal metabolic operation of cells (8, 10, 22). This places an important constraint on the transcellular transport of bulk quantities of calcium. Any excess calcium ion in the cytoplasm must be sequestered. Such sequestered concentrations of calcium might be expected to appear as discrete localizations.

Our results are supported by observations of other investigators. First, calcium has previously been found associated with apical and lateral cell membranes of intestinal epithelia of rats (27). Second, the association between calcium and goblet cell mucin has previously been reported in the rat ileum with the calcium-specific glyoxal bis (2-hydroxy-anil) (GBHA) method (21). Also, and of particular importance, an immunofluorescent antibody technique has been used to visualize the location of an intestinal calcium-binding protein (CaBP) in chicks at the cellular level (38). Due to its great affinity for calcium, CaBP would be half saturated even at concentrations as low as $10^{-6}\ \text{M}$ (6, 46). Thus, any accumulation of CaBP, even intracellular, should contain a substantial amount

of calcium. The distribution of CaBP is strikingly similar to the calcium distribution observed with EPMA in rats. CaBP was found within goblet cells and in association with the brush border surface coat (38). In addition, although not stressed by the authors, their results also show small fluorescent localizations of CaBP within the epithelial layer and the lamina propria.²

Nevertheless, in spite of these similarities between calcium and CaBP distributions, CaBP cannot be directly identified in the electron microprobe. In addition, several observations of the calcium distribution are at variance with the known distribution of CaBP. Whereas the concentration of CaBP decreases with age (45), calcium localizations are readily apparent in goblet cells of the duodenum of mature rats. Whereas the concentration of CaBP decreases distally along the intestine (36), the occurrence of calcium localizations within goblet cells remains constant. These observations suggest that other components of mucin besides CaBP are binding substantial amounts of calcium.

The importance of this latter observation is worth noting. Since the number of goblet cells increases distally down the intestine, there is a corresponding increase in the observed number of goblet cell calcium localizations. The greatest amount of calcium is absorbed not in the proximal intestine but in the distal region (24, 43). The pattern of calcium localizations is therefore correlated with the intestinal region where the greatest amount of calcium is absorbed.

The consistent association between calcium and mucus raises the question of mucus involvement in the transport process. One firm correlation between mucus and transport is CaBP. A relation between CaBP and calcium-transporting ability has clearly been demonstrated (37, 46, 44, 45, 47), and on the basis of its distribution CaBP would have to be classified as a component of mucus (38). However, Taylor and Wasserman have not specified exactly how CaBP participates in the calcium transport process. This may be due, in part, to the fact that products of goblet cells are usually described as having only "lubricative" and "protective" functions in the intestine (5, 17, 18, 34, 39). Goblet cell mucus has, however, been found to bind enough calcium to produce extracellular sites of calcification in gastric and intestinal adenocar-

² A. N. Taylor. 1972. Personal communication.

cinoma (3, 14, 35). Furthermore, mucus may be involved in the absorption of other material from the intestine such as vitamin B₁₂ (48) and horseradish peroxidase (12). Thus it is reasonable to entertain the possibility that goblet cell mucus is directly involved in the calcium transport process.

Path of Calcium Absorption

The microprobe resolution in this study does not permit calcium localization at an ultrastructural level; the path of calcium across absorptive epithelial cells can only be described in terms of general cellular areas. This is particularly restrictive for the many distributions seen near lateral cell membranes. First, it is not possible to ascertain whether these distributions are intracellular or extracellular, although inspection of fortuitous sections such as Fig. 16 would indicate that many are extracellular. Second, it is not possible to ascertain whether calcium passes through the tight junction, a path which seems likely for calcium movement. Clearly, calcium moving from lumen to lamina propria through absorptive cells encounters an energetics problem; calcium leaving the cell would have to surmount an enormous uphill electrochemical gradient. A pathway around the cell avoids this problem. Although Figs. 12 and 17 are not conclusive evidence that calcium moves through the tight junction and down an extracellular path, they are compatible with the existence of such a path for calcium.

As described in part II, most calcium localizations are observed along lateral cell margins, while some localizations appear at a supranuclear position before apparently moving to the lateral margins. This transcellular path of calcium is similar to that reported for a number of organic substances. Studies of fat absorption in the rat (7, 19), vitamin B₁₂ absorption in the dog (48), horseradish peroxidase absorption in adult (12, 23), neonatal, and fetal (23) rats, and γ -globulin antiferritin immunoglobulin in neonatal rats (30) all show these substances traversing the cell enclosed within membranes. The transport path is from apical cytoplasm to a supranuclear position, in some cases identified with the Golgi apparatus, and from there to the intercellular space and down this space to the cell base. In addition, the substances were also frequently observed along the entire lateral cell margins and intercellular spaces.

The similarity in transport pathway of these various organic substances suggests a fundamental

transcellular route. The fact that there is a similar path for calcium need not indicate an equivalent ionic pathway. Because protein shares this common route, one might expect the protein CaBP to follow a similar path. If calcium is transported across cells while complexed to CaBP, then one might expect to observe calcium following this organic pathway.

Effect of Vitamin D

As described in Results, part III, vitamin D increases the calcium content of localizations within goblet cells; within absorptive cells, there are no localizations in vitamin D-deficient rats and an increased number of localizations in D-replete rats. These observations are at variance with results from a previous electron microscope study (31). That study found frequent dense deposits of "intracellular bound calcium" concentrated in and limited to microvilli in vitamin D-deficient rats, with a paucity of similar deposits within mitochondria. In vitamin D-replete rats there was a decrease of deposits in the microvillus border and a concomitant increase within mitochondria. EPMA did not normally encounter calcium concentrations within the microvillus border in either vitamin D-deficient or replete rats. With our preparative procedures, the electron microscope did not reveal dense deposits within mitochondria (Figs. 22, 23). The calcium localizations which we observe in transit across cells are associated with lateral membranes or extracellular spaces rather than with mitochondria. Finally, we observe no phosphorus signal associated with calcium localizations. These observations argue against a relationship between mitochondria and calcium translocation.

The conflict between our results and the previous study may be due to the inadequacy of conventional electron microscope preparative techniques in preserving the distribution of soluble materials. The methods used in the electron microscope study have been shown readily to remove and redistribute calcium (10). Consequently, that study may not have localized calcium to the sites it occupied in the tissue before processing.

The results presented in Table III suggest the existence of another calcium transport mechanism in addition to the one represented by discrete localizations. It must be kept in mind, however, that EPMA measurements of such small amounts of calcium are likely to be subject to large errors.

Clearly, additional experiments using different methods and conditions will be required before further discussion can be justified.

In summary, the calcium transport process most evident to EPMA is that represented by discrete localizations. This process is compatible with models of transport which involve a transcellular calcium carrier or calcium-protein complex (44). This process is not directly compatible with the conventional concepts of a cationic pump, as frequently postulated for calcium transport (1, 25, 32, 33).

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